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Cadherin-11 is unique amongst cadherins in that it is exists as two alternatively spliced forms that are expressed together in the same cell. In year 1 of this grant we show that expression of wild-type cadherin-11, with or without co-expression of the C-terminal truncated splice variant, promotes epithelial differentiation of the cadherin-negative SKBR3 cell line. Exogenous wild-type cadherin-11 association with and membrane recruitment of β-catenin and p120 is unaffected by co-expression of the truncated variant. Cadherin-11 expressing cells exhibited modest changes in cell proliferation and no change in anchorage-independent growth. However, co-expression of wild-type cadherin-11 and the splice variant promoted a dramatic increase in the ability of SKBR3 cells and E-cadherin positive MCF-7 cells to traverse Matrigel-coated filters. Biochemical studies indicate that the truncated variant is secreted from the cell and enters a detergent-insoluble extracellular compartment. These data suggest that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11 positive breast cancer cells, perhaps by promoting cell-ECM interactions. In other studies a new antibody specific for cadherin-11 variant was developed and a series of cell lines expressing cadherin-11 ribozymes have been made.

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Introduction

The cadherins are a superfamily of transmembrane glycoproteins that mediate cell to cell adhesion in a variety of tissues (reviewed in (1), (2), (3)). Members of the classic cadherin subfamily possess five EC domains and a conserved cytoplasmic region. These molecules are responsible for formation of the adherens junction in most cell types. Upon calcium ion binding by the extracellular region, the EC domains change conformation, allowing lateral dimerization and subsequent cross-association between dimers on adjacent cells, generally in a homotypic fashion (i.e. E-cadherin to E-cadherin) (4). The cytoplasmic tail interacts directly or indirectly with "linker proteins," including β-catenin or plakoglobin, α-catenin, α-actinin, and vinculin. These proteins are thought to couple cadherins to the actin cytoskeleton in order to strengthen the adhesive force of the entire junctional complex (5).

The best-characterized classic cadherin is E-cadherin. Differential expression of E-cadherin has been implicated in several aspects of development, including cell sorting during gastrulation and tissue morphogenesis as well as the establishment of differentiated cell identity (for example, in the intestinal lumen) (6), (7). In addition, E-cadherin has been studied extensively with respect to its role as a putative tumor suppressor gene. Decreased E-cadherin expression has been correlated with metastasis and decreased survival in several different cancers, including breast carcinoma (8). However, E-cadherin loss is not an absolute predictor of tumor invasion or metastasis. Interestingly, increased expression of other cadherins, such as N-cadherin and P-cadherin, may also be associated with development or progression of breast carcinoma (9), (10).

Attention has been drawn recently to cadherin-11, which also may be involved in the promotion of carcinogenesis. Cadherin-11, or OB-cadherin, was originally identified in mouse osteoblasts (11); it was later found to be expressed in a variety of normal tissues of mesodermal origin, including areas of the kidney and brain (12). Although a type II cadherin (lacking a conserved HAV sequence), cadherin-11 is otherwise similar in structure to the type I cadherins N- and P-cadherin. In addition, the genomic structure of cadherin-11 exhibits a unique mRNA splice site, allowing for two forms of the protein to be expressed: "wild-type" cadherin-11, and a C-terminus-truncated variant (Figure 1a) (11). While the variant has an extracellular domain identical to that of wild-type cadherin-11, a frame-shift event at the point of splicing confers a unique cytoplasmic region with no homology to the cytoplasmic domain of any known cadherin (13). The function of the variant protein is unknown, but its sequence shows slight similarity to the src family of tyrosine kinases. In all situations so far examined, cadherin-11 and its variant form are expressed co-incidentally (14), (15).

Cadherin-11 mRNA is expressed in several types of cancer cells, including colon, gastric, renal cell, breast and osteosarcoma (16), (17), (18), (14),(15). In many instances cadherin-11 expression has been associated with more aggressive, dedifferentiated cancers, such as the signet-ring cell subtype of gastric carcinoma (18). In addition, we have previously shown cadherin-11 to be differentially expressed in more aggressive breast cancer cell lines (19).

However, no studies have examined the function of cadherin-11 in carcinoma cells. We have addressed this issue by first developing cadherin-11-specific antibodies. We then stably transfected SKBR3 cells, which do not normally express any other known cadherins, and MCF7 cells, which only express E-cadherin, with cadherin-11 and its variant form. We found that, in contrast to cadherin-11, which localizes to the cell membrane (14), the truncated variant is secreted from the cell and enters a detergent-insoluble extracellular compartment. In addition, cadherin-11, in the presence or absence of the truncated variant, can mediate the formation of a functional adherens junction complex, recruiting β-catenin, α-catenin, and $p120^{ctn}$ to the membrane. Finally, although expression of cadherin-11 and variant does not

dramatically alter anchorage-independent growth or cellular proliferation rates, it does cause significant changes in the invasive capacity of both cell types.

Materials and Methods:

Production of Fusion Protein Vectors: Portions of cadherin 11 cDNA were subcloned into the pGEX-2TK fusion protein expression vector (Amersham Pharmacia Biotech) as follows. Using RT-PCR, intracellular (WTIC) domains of cadherin 11 intact were amplified from total RNA derived from the MRC5 human embryonic lung cell fibroblast cell line (14). Oligonucleotide primers (For-5'-GCGGCGG*GATCCGCTGGGGAAGAAGACACAGAAG-3'; Rev-5'-CCGCCGG*AATTCTCTTGAGAACGCCAGACACAG-3') were designed to incorporate Bam HI and Eco RI restriction enzyme digest sites into the 5' and 3' ends, respectively, of the resulting cDNA fragments. RT-PCR reactions were performed with the following parameters: reverse transcription from 1.0ug total RNA using MMTV-RT (Life Technologies, Inc.) for 1 hour at 37°C with 0.2 um reverse primer was followed by addition of 0.2um forward primer and 30 PCR cycles (94°C-1min, 55°C-1.5 min, 72°C-2min). After isolation of appropriate PCR products by gel electrophoresis, the cDNA fragments were digested with Bam HI and Eco RI, gel purified, and ligated overnight into a BamHI/Eco RI-cut PGEX-2TK vector using T4 DNA ligase (Life Technologies). Clones were isolated, digested to ensure correct orientation of the insert, and subsequently sequenced to verify that no mutations occurred during amplification.

Production and Isolation of Polyclonal and Monoclonal Antibodies: pGEX-2TK-WTIC was transformed into DH5α bacterial cells, and GST-fusion proteins were made and purified using standard procedures. Two rabbits were immunized, given 4 injections one week apart, and then a fifth injection 2 weeks later. Rabbits were bled 10 days after the fifth booster injection and bled once more 10 days later, then given another injection 4 days later. Serum was tested by western blot to identify high titre animals. After the 10th immunization, rabbits were bled by collecting 25 ml and bleeding 3 times, 2 days apart and sacrificed. For purification, each 2 bleeds (25 ml each) following a single booster were pooled. An affinity column was made by conjugating the same peptide used for immunization to Aminohexane gel by Sulfo-SMCC. Serum was incubated with the gel and the column extensively washed. Purified antibodies were first eluted with KSCN, then with Glycine. The two fractions of antibodies were dialyzed, concentrated if necessary, then tested by immunoblot and/or immunoprecipitation and/or immunocytochemistry.

For production of monoclonal antibodies balb/C mice (8 weeks old) were immunized (i.p.) with recombinant fusion proteins mixed with complete Freund's Adjuvant and then boosted several times with the same antigen mixed with incomplete Freund's Adjuvant. Blood samples were collected and tested by ELISA and Western Blot. Mouse splenocytes from the best responders were fused with mouse myeloma cells (P3X63Ag8.653) using PEG. All hybridoma supernatant samples from the 96-well culture plates were screened by ELISA on plates coated with the appropriate fusion protein. Following expansion into 24-well plates, the ELISA-positive clones were further examined by Western Blot. All single-cell clones (subclones) were examined by, ELISA and western blot to confirm their production of the antibody of interest. Final subclones were isotyped by Mouse MonoAb ID Kit (Zymed, Cat#90-6550). Ascites from clone 5B2H5 was produced in Balb/C mice and purified using a Protein A column.

Characterization of Antibodies: Characterization of antibodies was performed by western blot analysis of several cell lines that expressed varying combinations of exogenous or endogenous cadherin 11 and variant, E-cadherin, and N-cadherin. Briefly, SKBR3 cells (express no known cadherins) and MCF7 cells (express E-cadherin) were transiently transfected with cadherin 11 or variant cDNA. These cells, as well as parental SKBR3s and MCF7s, MDA-MB 231s (express Cad-11 intact and variant), HS578Ts (express

cadherin-11 and variant and N-cadherin), and MDA-MB 435s (express N-cadherin but not cadherin-11) were lysed in NP-40 lysis buffer (1% Nonident P-40, 150 mM NaCl, 50mM Tris pH 8.0, 1mM NaVanadate, 50mM NaF, and protease inhibitors [Boehringer Mannheim]). The soluble fractions were isolated by centrifugation at 12,000 g for 10 minutes at 4°C, diluted in sample buffer, and run under denaturing conditions on 4-12% Tris-Glycine gels (Novex Corp.). Following transfer to nitrocellulose and blocking for 1hr at room temperature in 5% non-fat milk in PBST, membranes were incubated with each antibody at varying concentrations, rinsed, and incubated with appropriate secondary antibody conjugated to peroxidase (Jackson ImmunoResearch). Blots were visualized using ECL (Amersham). All cadherin 11 antibodies were also used in immunocytochemistry, immunoprecipitation, and immunohistochemistry assays to further determine specificity as well as efficacy in these techniques.

Cell Culture and Production of Stable Transfectants: SKBR3 cells were obtained from the ATCC (Rockville, MD) and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 10% FBS (Biofluids). Cells were transfected with pCXN2-cad11-intact and/or pCXN2-variant (gifts from Akira Kudo, Tokyo Inst. of Tech), or pCDNA3-CAT (as a control) and equal amounts of the puromycin-resistance plasmid pHA262pur (a gift from Dr. H. te Riele, Netherlands Cancer Institute) using the calcium phosphate method. Resistant pools were subsequently selected in 1ug/ml puromycin for 4-6 weeks, further enriched by differential trypsinization, and characterized by western blot, RT-PCR, and/or immunocytochemistry.

MCF7 cells were obtained from the ATCC (Rockville, MD) and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 5% FBS (Biofluids). Cells were transfected with pCXN2-cad11-intact and/or pCXN2-variant, or pCDNA3-CAT (as a control) using Lipofectamine Plus (Life Technologies, Inc.) and selected in 0.5mg/ml G418 for 4-6 weeks. Clonal populations of cells were obtained by plating to a limiting dilution in 0.5 mg/ml G418; subsequent clonal populations were screened by immunocytochemistry and western blot analysis.

Antibodies: The following primary antibodies were used for immunocytochemistry, immunoprecipitation, and immunoblotting: monoclonal anti-cadherin 11 clone 5B2H5 and polyclonal anti-cadherin 11 WTID (Zymed Laboratories), both of which were raised against the intracellular domain of cad11 and hence recognize the intact cad-11 alone; monoclonal anti-cadherin 11 (a gift from MJ Bussemakers, (228)) and monoclonal anti-cadherin 11 113H (a gift from the ICOS Corp.), both of which recognize the extracellular domain of cadherin-11 and variant; monoclonal anti-β catenin (Transduction Laboratories); polyclonal anti-β catenin SHB7 (a gift from D. Rimm); monoclonal anti-$p120^{ctn}$ (Transduction Laboratories); and monoclonal anti-$p120^{ctn}$ clone 12F4 (a gift from A.B. Reynolds).

Immunocytochemistry and Microscopy: Cells were plated on sterile 18mm glass coverslips and allowed to adhere at least 48 hours prior to fixation in 2% paraformaldehyde (20 minutes), with subsequent permeabilization in 0.2% Triton-PBS (5 minutes). Cells were blocked for 1 hour at room temperature in 3% ovalbumin-PBS, and incubated with the appropriate antibody diluted in 6% normal goat serum-PBS for 1 hour at room temperature. After 3 x 3 minute washes in PBS, cells were incubated with the appropriate secondary antibodies conjugated to either Texas Red (Jackson ImmunoResearch) or FITC (Kirkegaard & Perry) for 1 hour at room temperature in the dark. All secondary antibodies were diluted 1:200 in 6% normal goat serum-PBS. For double staining, polyclonal primary and anti-rabbit secondary antibodies were applied first, followed by monoclonal primary and anti-mouse secondary antibodies. After the final 3 x 3 minute washes in PBS, coverslips were mounted on slides with Vectashield. All fluorescence and Nomarski Interference Contrast images were digitally captured using an Olympus Fluoview Confocal Microscope.

Immunoblotting and Immunoprecipitation: Cells were solubilized in ice-cold 1% NP-40 buffer solution (1% NP-40, 1250 mM NaCl, 50mM Tris pH 8.0) containing 1mM NaVanadate, 50mM NaF, and complete protease inhibitors (Boehringer Mannheim). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4° C to remove the NP-40 insoluble material. After addition of 2X sample buffer (4% SDS, 120mM Tris pH 6.8, 20% glycerol) to the NP-40 soluble fraction and 1X sample buffer to the insoluble pellet, samples were boiled and Bio-Rad protein assays were performed to determine total protein content. After addition of reducing agent, the samples were again boiled, and equal total protein was loaded on 3-8% NuPage tris-acetate gels (Invitrogen Corporation) unless otherwise indicated. Proteins were blotted to nitrocellulose (Schleicher & Schuell) and blocked for 1hour at room temperature or overnight at 4° C in 5% milk-PBST. After incubation with appropriate primary and secondary antibodies each for 1 hour at room temperature, blots were treated with ECL reagent (Amersham) and exposed to film. Blots were sometimes stripped (62.5 mM Tris pH 7.5, 2% SDS, 1.7% B-mercaptoethanol for 30 minutes at 50°C), reblocked, and reprobed with new primary and secondary antibody. Alternatively, cells were lysed in ice cold 1X LDS sample buffer (Invitrogen Corporation), scraped, passed through a 27 gauge needle, and boiled in the presence of a reducing agent for 30 minutes before gel electrophoresis and immunoblotting as described.

For immunoprecipitation, lysates were obtained as described above. Lysates were first precleared with 50 ul Protein G-sepharose beads (Zymed) alone for 1 hr at 4° C. The beads were centrifuged, removed, and appropriate precipitating antibodies were added to lysates for 1 hour with rocking at 4° C; 50µl of new beads were then added with rocking for an additional 2 hours at 4°C. After three washes with ice-cold lysis buffer, sample buffer and reducing agent were added to precipitated proteins and beads and boiled for 10 minutes; samples were subsequently analyzed by immunoblotting as described above.

Reporter Assays 12-well plates were seeded at 10^5 cells/well 24-48 hours prior to transfection. Cells were transfected with TOPFLASH reporter (indicates LEF reporter activity), pCXN2-cadherin-11wild-type and pCXN2-cadherin-11 variant or pCDNA3-CAT (control), and the TK-renilla luciferase plasmid (Promega Inc.) to control for variations in transfection efficiency. After lysis, luciferase and renilla activities were read on a standard luminometer using the Dual-Reagent Luciferase Assay Kit (Promega, Inc.). Luciferase values were normalized to renilla values and plotted using Sigma Plot™. Each experiment was performed in triplicate at least three independent times, with error bars representing standard deviation.

Proliferation Assays: WST-1 assays (Boehringer Mannheim) were performed as indicated by the manufacturer. Briefly, 1000-3000 cells of each population were plated in triplicate in 96 well plates on Day 1. WST-1 readings were taken on alternate days beginning with Day 0 on an optical densitometer. For analysis (Sigma Plot™), data for each population was plotted relative to the mean Day 0 value to account for variance in plating efficiency, with error bars representing standard deviation. All experiments were performed independently at least three times.

Soft Agar Assays: Soft agar assays were performed as described (20). Briefly, cells were plated in 6-well plates at 5 x 10^5 cells/well in a 0.3% agar suspension (SeaKem) on a 0.6% agar cushion, with one ml DMEM + 5% FBS covering the cells. The cells were incubated at 37°C + 5% CO_2 and media was carefully changed every 3-4 days. After 2 or 3 weeks, colonies greater than, 140 um in diameter, were scored by an Omnicon 3600 Colony Counter; data was subsequently analyzed and graphed on Sigma Plot™. All experiments were performed in triplicate at least three independent times; error bars represent standard deviation.

In vitro Invasion assays: Invasion assays were performed as previously described (21), (9). Standard Boyden chambers were prepared by placing NIH3T3-conditioned media (24 hours, DMEM + 50ug/ml

ascorbic acid) in the bottom well of the chamber as a chemoattractant. After coating a 12-μm pore size polycarbonate filter (Poretics, Inc.) with 10ug matrigel, $3x10^5$ cells in DMEM with 0.1% BSA were placed in the upper chamber and incubated for 16 hours at 37°C. Membranes were then removed, cells fixed in 25% methanol with 0.5% crystal violet, and remaining cells wiped from the upper surface of the membrane with a damp cotton swab. Quantification of cells on the bottom of the membrane was performed by counting the number of cells/field in five random fields per membrane; the fields were then averaged. Bars represent the mean of each population over three membranes with error bars representing standard deviation. Each experiment was performed at least three independent times.

Results:

Cadherin 11 Antibody Production and Characterization: To characterize the monoclonal and polyclonal antibodies raised against cadherin-11, we first determined their specificity by western analysis. Lysates from MDA-MB 231 cells were evaluated because these cells express both forms of cadherin-11 (14). In addition, we examined lysates from MCF7, HS578T, and MDA-MB-435 cells, which express E-cadherin, N-cadherin and cadherin-11, and N-cadherin, respectively (21), (14). Cadherin-11 is relatively homologous to E- and N-cadherin; anti-cadherin-11 antibodies, especially those raised against the intracellular domain, were therefore most likely to cross-react with these proteins. Finally, in order to determine whether antibodies could recognize exogenously expressed cadherin-11, we evaluated lysates from both SKBR3 cells and MCF7 cells transiently transfected with cDNA encoding the wild-type cadherin-11 or variant protein.

One monoclonal antibody (5B2H5) to the intracellular domain of cadherin-11 intact was reactive by western blot, indicated by the ~115kD species (Figure 1b). 5B2H5 did not react with the variant form, nor with any proteins in the MCF7 or MDA-MB 435 lanes (E-cadherin and N-cadherin, respectively), indicating that it was specific to the wild-type form of cadherin-11. In addition, the only band seen in the HS578T lane is 115kD, indicating that it is cadherin-11; N-cadherin runs at approximately 140kD. The 5B2H5 clone was subsequently found to be useful for immunocytochemistry, immunoprecipitation, and immunohistochemistry and is available commercially as Zymed 32-1700 (clone 5B2H5).

One polyclonal antibody raised against the intracellular domain of wild-type cadherin-11 intact was also analyzed and found to recognize not only cadherin-11, but also E-cadherin and N-cadherin (Figure 1c). This antibody (pWTID1) is also useful for immunocytochemistry; its utility in immunoprecipitation or immunohistochemistry was not investigated due to its cross-reactivity with E- and N-cadherin. This pan-cadherin antibody is available commercially as Zymed 71-7600 (cadherin-11 WTID1). We were not successful in generating monoclonal or polyclonal antibodies specific to the cadherin-11 variant intracellular domain.

Characterization of Stable Transfectants Expressing Cadherin 11: Both SKBR3 and MCF7 breast cancer cells are relatively well differentiated and moderately invasive in in vitro invasion assays. Since SKBR3 cells have a homozygous deletion of the E-cadherin gene (22) and do not express other cadherins, they represent a suitable system for determining whether or not cadherin-11 could act as a functional adherens junction molecule, in addition to whether or not it could induce a phenotypic change. The MCF7 cell line, which expresses E-cadherin alone, is an appropriate model to determine whether exogenous cadherin-11 expression interferes with E-cadherin function, as well as induce a more proliferative or invasive phenotype in the presence of E-cadherin.

Cells were co-transfected with vectors containing the full-length cDNA coding for the intact and/or variant forms of human wild-type cadherin-11 (pCXN2-cad11 and pCXN2-cad11variant), or empty vector containing the chloramphenicol transferase (CAT) cDNA for control, with or without a second puromycin resistance plasmid (pHA262pur). Pooled stable populations expressing cadherin-11, or

both cadherin-11 and the cadherin-11 variant, or CAT were obtained either by selection with G418 or puromycin.

MCF7 cells were transfected with cadherin-11 and variant or CAT as described above; selection with G418 resulted in pooled populations 40-90% positive for cadherin-11. In order to obtain populations in which 100% of cells expressed the appropriate protein(s), we selected clones by limiting dilution in G418 with subsequent screening by immunocytochemistry (Figure 2a). Further characterization by RT-PCR analysis revealed production of the cadherin-11 variant in all clones (Figure 2b).

Expression of Cadherin-11 Results In Morphological Changes in SKBR3 cells: SKBR3 cell lines were co-transfected with the puromycin resistance plasmid pHA262pur and cadherin-11 and/or cadherin-11 variant or CAT cDNA. Selection with puromycin produced populations of SKBR3 cells transfected with cadherin-11 and the cadherin-11 variant that were 90-100% positive without the need for further sorting (Figure 2a). Because we had no antibody specific to the variant form of cadherin-11, cells were further characterized by RT-PCR analysis (Figure 2c). Using primers specific for the variant mRNA species, it was clear that only SKBR3 cells transfected with the variant cadherin-11 cDNA expressed the variant mRNA (MDA-MB 231 cells were used as a positive control). SKBR3 cells transfected with only wild-type cadherin-11 and selected in puromycin were approximately 40% positive for cadherin-11; this was further enriched to ~100% by differential trypsinization, as SKBR3 cells expressing cadherin-11 adhered to plastic much more efficiently than control cells. Neither CAT transfected nor parental SKBR3 cells express cadherin-11.

In SKBR3s, the expression of cadherin-11 alone or cadherin-11 and cadherin-11 variant produced a profound change in morphology (Figure 3a). Parental SKBR3 cells (no known cadherins) are weakly attached to one another, resulting in loose aggregates of cells that detach easily from one another and from tissue culture treated plastic, especially when grown to high density. Cells expressing cadherin-11 or cadherin-11 and the variant formed closely adherent islands of cells; some of these islands grew quite large, incorporating hundreds of cells (Figure 3a). These foci were significantly more difficult to dislodge from plastic with trypsinization than parental or SK-CAT cells, suggesting an increased ability of these cells to adhere to one another and to the extracellular substrate. No differences were observed in the morphology or trypsinization properties of cells expressing cadherin-11 alone when compared to those expressing both cadherin-11 and the variant form.

MCF7 clones expressing cadherin-11 and the variant form did not exhibit any noticeable changes in morphology when compared to control cells (data not shown). This is probably because MCF7 cells already express E-cadherin, which confers strong cell-cell adhesion; it was unlikely that cadherin-11 expression would increase this (21). However, it is significant that expression of cadherin-11 and variant did not appear to disrupt this adhesion.

Cadherin 11 expression results in the assembly of adherens junction components: As discussed earlier, cadherin-11 is generally expressed in mesenchymal cells or cells with a mesenchymal phenotype, such as invasive cancer cells. These types of cells do not usually form stable adherens junctions. However, the morphological changes that accompany the expression of cadherin-11 in SKBR3 cells suggest that this cadherin, like E-cadherin, can not only promote homotypic cell adhesion but can also mediate the formation of adherens junctions. We previously showed that β-catenin interacts with cadherin-11 in cells that express both proteins endogenously (14). In contrast, Reynolds *et al.* suggested that another catenin and adherens junction component, $p120^{ctn}$, does not interact with cadherin-11. They demonstrated that $p120^{ctn}$ does not localize to the membrane in MDA-MB 231 cells, which only express one cadherin, cadherin-11, in the membrane at sites of cell-cell contact (23). We confirmed these results (not shown). The localization of $p120^{ctn}$ in the cytoplasm of MDA-MB 231 cells, instead of at the

membrane with cadherin-11, suggested that these molecules might not interact with one another. We therefore tested the ability of cadherin-11 to recruit members of the adherens junction complex to the membrane of SKBR3 cells expressing cadherin-11 alone or cadherin-11 and variant.

Cadherin-11 expressing SKBR3 cells were stained for cadherin-11, β-catenin and $p120^{ctn}$ and visualized by confocal microscopy (Figure 3b). These experiments were carried out on puromycin selected pooled stable expressors before final enrichment (see Materials and Methods). Consequently, not all cells express cadherin-11; the negative cells act as internal controls in this instance. Cells that are positive for cadherin-11 (red) express the protein at the membrane at sites of cell-cell contact; there is no cadherin-11 at the edges of cells not contacting other cells. Staining for β-catenin (green) reveals that cadherin-11 recruits β-catenin protein to the membrane. Co-localization of the two proteins is clear in the merged image. Parental SKBR3 cells exhibit barely detectable levels of β-catenin only in the cytoplasm, similar to the cadherin-11 negative cells in the left margin of the image (21). Consequently it is likely that cadherin-11 is stabilizing β-catenin (i.e. preventing its degradation) by recruiting it to the cell membrane. In other systems expression of E-cadherin can recruit β-catenin to the membrane and reduce its ability to activate TCF-reporters (24). Figure 4 shows that expression of cadherin-11 can also reduce the ability of β-catenin to activate TCF-reporters.

Expression of cadherin-11 also resulted in a marked relocation of $p120^{ctn}$ to the membrane (Figure 3b). Finally, concurrent expression of the variant form of cadherin-11 did not appear to stimulate or impede the ability of cadherin-11 to recruit either catenin to the membrane (Figure 3c). Similar experiments were also performed in MCF7 cells. These cells already express E-cadherin and co-expression of cadherin 11 and variant did not alter the distribution of β-catenin or $p120^{ctn}$ (not shown).

In order to verify that cadherin-11 exists in a complex with both β-catenin and $p120^{ctn}$, instead of simply co-localizing in the same vicinity, immunoprecipitation experiments were performed (Figure 5). Both β-catenin and $p120^{ctn}$ were present in cadherin-11 immunoprecipitations from cadherin-11 expressing SKBR3 cells. No $p120^{ctn}$ was detected in cadherin-11 immunoprecipitates from SK-CAT control cells. In addition, the β-catenin that precipitated with cadherin-11 has a slower electrophoretic mobility than β-catenin found in the cell lysate. Finally, re-probing of these blots determined that a third catenin, α-catenin, is present in the complex, and also appears to be of a slightly higher molecular weight than protein found in the cell lysate (Figure 5a).

We also wished to determine whether the expression and activity of cadherin-11 and variant proteins might affect or be affected by the presence of E-cadherin. We therefore immunoprecipitated either cadherin-11 or E-cadherin from parental MCF7 cells and cadherin-11 expressing MCF7 cells and blotted for the same catenins described above (Figure 5b,c). We found that neither cadherin-11 nor E-cadherin prevented the other from associating with $p120^{ctn}$, β-catenin or α-catenin. In addition, $p120^{ctn}$ appeared to immunoprecipitate more readily with cadherin-11 than with E-cadherin. Finally, expression of cadherin-11 does not appear to alter the steady-state levels of E-cadherin expression.

Effects of Exogenous Cadherin 11 on Cellular Proliferation: In order to determine whether exogenous expression of cadherin-11 might affect the cellular phenotype, we assessed several indicators of cellular behavior, including proliferation rate, anchorage independent growth, and invasive activity. The rate of cell proliferation was assessed by, plating cells at varying densities and monitoring their growth for 6-14 days using the WST-1 assay, which measures cellular metabolic activity. Expression of wild-type cadherin-11 in SKBR3 cells led to a moderate change in the proliferation rate of cells (Figure 6a). At the peak of the proliferation log phase, cadherin-11 expressing SKBR3 cells exhibited approximately 150% the growth rate of SK-CAT cells. In SKBR3 cells expressing both cadherin-11 and

variant proteins the growth rate was approximately 60% that of control cells. The difference in cell proliferation between the two cadherin-11 expressing cell populations at log peak was significant, at $p<0.001$. However, co-expression of cadherin-11 and variant proteins did not alter the peak of the proliferation log phase for these cells. Instead, it took them longer to reach log phase than the control cells (14 days versus 6 days). These data suggest that expression of cadherin-11 alone may confer a slight proliferative advantage to SKBR3 cells, which appears to be reversed by concomitant expression of the variant protein. It should be noted that although the observed changes in proliferation rate assessed by the WST assay were repeatable and statistically significant they were not large. For example, we did not notice in the daily management of the cells that they needed to be passaged at different times. In MCF-7 cells no consistent differences in the rate of proliferation could be observed amongst the various control and cadherin-11 expressing cells (data not shown).

Exogenous Cadherin 11 Does Not Affect Anchorage Independent Growth: The ability of cells to grow in soft agar is not always related to changes in the proliferation. Consequently, it is possible that although cadherin-11 expression does not have a major effect on cell growth it may affect the ability of cells to grow in an anchorage-independent assay. The effect of cadherin-11 expression on anchorage independent growth was examined in both SKBR3 and MCF7 cells by culturing them on a cushion of soft agar for two weeks. We found that the ability of SKBR3 and MCF7 cells to grow in an anchorage independent fashion was neither stimulated nor reduced by expression of cadherin-11 or by co-expression of cadherin-11 and variant proteins (Figure 6b,c).

Exogenous Cadherin 11 Affects Invasive Activity: We have previously found that cadherin-11 is expressed in breast cancer cell lines displaying a more aggressive and invasive phenotype. We therefore wished to determine whether cadherin-11 expression might actually have a causal role in the acquisition of invasive capacity. In order to do so, we tested the ability of cadherin-11 expressing cells to invade a Matrigel-coated membrane in a standard Boyden Chamber assay. Equal numbers of control and cadherin-11 expressing cells were trypsinized and then plated onto Matrigel-coated filters above a well filled with chemoattractant-enriched media. Cells were then incubated for 16 hours to assess their ability to move through the Matrigel towards the chemoattractant on the other side of the membrane. We found that expression of cadherin-11 in SKBR3 cells led to a five-fold reduction in invasive capacity (compared to control CAT-expressing or parental cells) that was statistically significant, at $p<0.05$ (7). In contrast, cells expressing cadherin-11 and the variant protein invaded at twice the rate of control cells ($p<0.05$) and ten times faster than cells expressing cadherin-11 alone ($p<0.001$). Despite these clear differences in invasive behavior, both populations of cells were morphologically indistinguishable when viewed by phase contrast microscopy (Figure 3a).

We next hypothesized that transient expression of cadherin-11 variant in cells stably expressing wild-type cadherin-11 alone might reverse their reduced invasive capacity. To test this we transiently transfected cDNA encoding cadherin-11 variant or pcDNA-CAT (control) into cadherin-11 expressing SKBR3 cells, and after 24 hours trypsinized them and assessed their invasive capacity as before. In addition, after cells were trypsinized, some were plated on coverslips, and subsequently fixed and stained for cadherin-11 at the conclusion of the 16-hour invasion assay. We found that transient introduction of cadherin-11 variant into cadherin-11-expressing SKBR3 cells did increase their invasive capacity approximately two-fold, although this did not quite reach statistical significance ($p=0.08$). (Figure 8a,b). However, this experiment is limited by the transfection efficiency of the SKBR3 cells. In this experiment only 10% of the wild-type cadherin-11 expressing SKBR3 cells also expressed the transfected cadherin-11 variant (Figure 8b). Consequently it is likely that the two-fold increase in invasive capacity could actually reflect the increased invasive capacity of most of the cells that expressed both cadherin-11 forms.

Nieman et al. showed previously that expression of cadherin-11 in the E and P-cadherin expressing line BT20 slightly increased the invasive activity of the cells (25). It was not clear if both cadherin-11 and the variant protein were expressed in these experiments, but it is possible that the effects of cadherin-11 on invasion might be different in cells that express other cadherins. To further confirm that cadherin-11 plays a role in the regulation of cell invasion in cells that already express other cadherins we repeated these experiments in MCF7 cells, which express E-cadherin. Co-expression of cadherin-11 and cadherin-11 variant increased the invasive capacity of MCF7 cells 7 fold compared to parental cells or cells expressing CAT (Figure 7). This increase in invasive capacity occurs despite expression of E-cadherin in these cells, indicating that cadherin-11 may in some way alter the tumor suppressive activities of E-cadherin, or that its ability to bring about an invasive phenotype is independent of E-cadherin.

Localization of the cadherin-11 variant: The marked effects of expression of the variant cadherin-11 on the invasive behavior of SKBR3 cells and MCF7 cells prompted us to re-examine its localization in the cell. Because we were not successful in generating antibodies that specifically recognized the cadherin-11 variant we were forced to rely on antibodies that detected both cadherin-11 forms. In earlier experiments we found that a protein corresponding to the predicted size of the variant form (~85 kD vs. 115 for wild-type cadherin-11) was present in NP40 cell lysates from cells expressing endogenous cadherin-11(14). However, in the present study we found that cells transfected with wild-type cadherin-11 cDNA but not the variant cDNA also expressed this protein (Figure 9a). Because the variant form is a product of alternative splicing of the primary transcript it cannot have been present in these cells; we hypothesize that the ~85 kD protein is actually a degradation product of wild-type cadherin-11. We found that it was difficult to consistently detect the variant protein in cells that had been transfected with variant alone or variant plus cadherin-11 by immunocytochemistry or western blot of NP40 extracts even though there was abundant expression of the variant mRNA. This led us to speculate that the loss of 2/3 of the transmembrane domain in the variant form of cadherin-11 might allow it to be secreted from the cells. To test this, cells were transiently transfected with CAT, cadherin 11 or variant cDNA. In some experiments we incubated the cells for 24 hours at 22°C to prevent protein secretion and then processed them for immunocytochemistry (26). In other experiments, 12 hours after transfection the cells were placed in low-serum media (0.1%) for 72 hours. Media was then collected and concentrated 40-fold in the presence of protease inhibitors. Immunoprecipitation of cadherin-11 variant from media was carried out using two antibodies that recognize the extracellular domain common to cadherin-11 and its variant; neat media was also analyzed for variant protein by western analysis. In addition, the cells were lysed in either sample buffer or NP-40 lysis buffer to ensure adequacy of transfection.

In cells that were subjected to a low temperature secretion-blocking step (incubated at 22°C) we could clearly detect the variant protein inside the transfected cells using immunocytochemistry (Figure 9e). Since it was difficult to detect the cadherin-11 variant in cells in which secretion was not blocked, we interpret this result as showing that the variant form was indeed normally secreted. However we were unable to detect the variant protein in conditioned medium by straight western analysis or by western analysis on immunoprecipitated material (Figure 9b). We next hypothesized that, like wnts and FGFs, the cadherin-11 variant might actually be secreted but then immediately sequestered in an NP-40 insoluble form in the extracellular matrix. To test this we transiently transfected MCF7 cells with cadherin-11, cadherin-11 variant or control (CAT) cDNA. Next, instead of making an NP-40 lysate we subjected the cells to LDS lysis, followed by scraping, shearing, and boiling. This process results in the solubilization of all cellular and extracellular matrix components. Remarkably this procedure allowed visualization of the transfected variant protein, seen clearly in the middle lane (Figure 9c). Note the degradation product of cadherin-11 of almost identical molecular weight but in the NP-40 soluble fraction. Further analysis of NP-40 insoluble fractions confirmed that whereas cadherin-11 is predominantly in the NP-40 soluble fraction, the cadherin-11 variant is predominantly in the NP-40 insoluble, LDS-soluble fraction (FIG 9d). Although this fraction includes intracellular cytoskeletal and associated proteins as well as extracellular

matrix, the secretion experiments discussed above together with our difficulty detecting the variant protein within the cell strongly suggest that the cadherin-11 variant is secreted and subsequently bound to the extracellular matrix deposited by the cells.

Generation of a cadherin-11 variant antibody: None of the antibodies used in the preceding studies can specifically detect the cadherin-11 variant without cross-reacting with cadherin-11 itself. We have now made a polyclonal antibody directed against the unique intracellular region of the cadherin-11 variant. Preliminary results show that this antibody recognizes a band of the appropriate size in cadherin-11 expressing MDA-231 cells but not in MCF-7 cells, which do not express cadherin-11 (Figure 10). In years 2 and 3 we will further characterize this antibody.

Generation of cadherin-11 ribozyme expressing cell lines: An important aspect of aim 2 of the proposal is the use of MDA-231 cells, which do not express cadherin-11. To carry out this aim we have generated a number of lines that express a cadherin-11 ribozyme (directed against a region common to both cadherin-11 and cadherin-11 variant). Several of these lines express little to no cadherin-11 (Figure 11). These lines will be further characterized in years 2 and 3.

Key Research Accomplishments:

1. Demonstration that cadherin-11 can either promote cell-cell adhesion or promote invasion depending upon the concommitant expression of the cadherin-11 variant.

2. Generation of a cadherin-11 variant antibody:

3. Generation of cadherin-11 ribozyme expressing cell lines:

Reportable Outcomes:

An alternatively spliced cadherin-11 regulates human breast cancer cell invasion. Paper submitted

Conclusions:

The acquisition of an invasive or metastatic phenotype by tumor cells is a complex process involving changes in a number of cellular characteristics and behaviors. Changes in cell adhesion are thought to be particularly important (27). The cadherins are a family of adhesion molecules that have been investigated extensively with respect to their roles in suppression of invasion (28). More recently, expression of certain cadherins, including N-cadherin, has been associated with increased invasive activity in tumor cells (9). Another cadherin, cadherin-11, was recently associated with breast cancer cell lines that have a more invasive, poorly differentiated phenotype (14). In the present study we show that cadherin-11 with or without expression of the variant form can recruit components of the adherens junction to the membrane and result in a marked epithelioid differentiation of SKBR3 cells. Expression of cadherin-11 and its variant have little effect on cell proliferation and anchorage-independent growth but they markedly alter the invasive ability of breast cancer cells even in the presence of E-cadherin. Our demonstration that expression of the cadherin-11 variant changes the function of cadherin-11 from one of conferring cell-cell adhesion to one of promoting invasion is particularly important. To address this aspect further we have now made an antibody that only recognizes the cadherin-11 variant and have generated cell lines in which cadherin-11 expression has been abolished.

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Appendix:

Figure Legends:

Figure 1 (A) The genomic sequence of cadherin-11 is depicted at the junction between exons 13 and 14, which corresponds to amino acid 632 in the transmembrane region of wild-type cadherin-11. Cadherin-11 is produced when the intron between exons 13 and 14 is removed, and the subsequent mRNA is translated. The variant protein arises when a portion of the intervening intronic sequence is alternatively spliced between exons 13 and 14 and subsequently translated. The splice event produces a frameshift and early truncation of the protein due to a stop codon 179bp after the splice site. The resulting variant protein is therefore identical to wild-type cadherin-11 in its extracellular region and the 5' transmembrane region (1). 3' to the splice site, however, the variant (3) exhibits no homology to the intracellular domain of cadherin-11 (2) (*adapted from (13)*). (B) NP-40 lysates from different breast cancer cell lines expressing endogenous or exogenous cadherins were evaluated by western analysis to determine the specificity of monoclonal 5B2H5 antibody. SKBR3 parental cells express no known cadherins; MCF7 cells express only E-cadherin; MDA-MB-231 cells express cadherin-11; HS578T cells express N-cadherin and cadherin-11; and MDA-MB-435 cells express N-cadherin alone. In addition, SKBR3 and MCF7 cells were transiently transfected with cDNA encoding cadherin-11 or variant. A distinct 120kD band is recognized in those cells expressing cadherin-11. Note that the antibody does not recognize the variant form, nor cross-react with N-cadherin or E-cadherin, the two cadherins most closely related to cadherin-11. Monoclonal antibody 5B2H5 is now offered by Zymed Laboratories as catalog number 32-1700. (C) The polyclonal antibody WTID1 was characterized by analyzing lysates from MDA-MB-231 cells (cadherin-11), MCF7 cells (E-cadherin) and MDA-MB-435 cells (N-cadherin) and found to recognize all three cadherins. It is therefore a useful pan-cadherin antibody, and is also distributed by Zymed Corporation as catalog number 71-7600.

Figure 2 (A) SKBR3 cells were co-transfected with cadherin-11 without or with variant (SK-INT or SK-IV, respectively) or pcDNA-CAT (SK-CAT) as a control, and a puromycin resistance plasmid; selection resulted in pooled populations expressing the protein(s) of interest. MCF7 cells were transfected with cadherin-11 and variant (IV) or pCDNA-CAT and selected with G418 before selecting clonal populations. To verify protein expression, cells were plated on coverslips, allowed to attach and grow for at least 48 hours, fixed, and permeabilized. All cells were stained using the Bussemakers' antibody at a 1:200 dilution (recognizes the extracellular domain of cadherin-11) and a secondary goat anti-mouse antibody conjugated to Texas Red. Transfected SKBR3 and MCF7 cells expressed cadherin-11 at the membrane, while neither control nor parental cells exhibited any signs of membrane staining. (B) RT-PCR was used to verify production of cadherin-11 variant mRNA. Using primers that amplify a 194-bp region specific to the variant alone, reverse transcription and subsequent PCR cycles were performed on DNAse-I-digested total RNA isolated from MCF7 clones stably expressing CAT or cadherin-11 and variant (MCF7-CAT and MCF7-IV). Note that control CAT cells do not express the 194-bp PCR product. MDA-MB-231 cells (+), which express cadherin-11 and variant mRNA (14), were used as a positive control. (C) RT-PCR was performed in order to ensure that the stably transfected SKBR3 cells expressed cadherin-11 variant mRNA. Visualization by agarose gel electrophoresis demonstrated that only SKBR3 cells stably transfected with variant cDNA (SK-IV) were transcribing variant message, while control cells and those expressing wild-type alone did not (SK-CAT and SK-INT).

Figure 3 (A) Examination of SKBR3 populations by light microscopy reveals that cells stably expressing wild-type cadherin without or with the variant form are markedly changed in appearance compared to control cells. Stable expressors of cadherin-11 with or without variant (SK-INT and SK-IV, respectively) appear to form "syncytia" or colonies of cells with apparently tight cell-cell adhesion. Note that there

does not appear to be any morphological distinction between cells expressing cadherin-11 and variant versus cells expressing cadherin-11 alone. (B) SKBR3s expressing cadherin-11 were grown on glass coverslips, fixed and permeabilized before immunocytochemistry against cadherin-11 and either β-catenin or $p120^{ctn}$. Cells were stained for β-catenin using a polyclonal SHB7 antibody (1:1500) with a FITC-conjugated secondary antibody (green), and then for cadherin-11 using the monoclonal Bussemakers antibody and a Texas Red-conjugated secondary antibody (red). Cadherin-11 is present at sites of cell-cell contact at the membrane; β-catenin co-localizes with cadherin-11 at these sites. Note that cells that are negative for cadherin-11 do not exhibit β-catenin protein. Cells were also separately stained for cadherin-11 using the pWTID antibody (1:1000) with a FITC-conjugated secondary antibody, and then for p120 with the monoclonal 12F4 antibody (1:1000) and a Texas Red-conjugated secondary antibody. $p120^{ctn}$ co-localizes with cadherin-11 in a manner similar to β-catenin. Of note, the 12F4 antibody recognizes all four p120 isoforms. (C) Double-labeling for cadherin-11 and either β-catenin or $p120^{ctn}$ was performed in SKBR3 cells stably expressing cadherin-11 and variant proteins as described above. The presence of cadherin-11 variant does not affect the ability of wild-type cadherin-11 to recruit either catenin to the membrane.

Figure 4 SKBR3 cells were transiently transfected with a β-catenin/LEF reporter construct, TOPFLASH. The TOPFLASH plasmid contains repeats of the DNA sequence to which the β-catenin/LEF complex binds, driving the expression of luciferase. TOPFLASH luciferase output therefore measures β-catenin signaling activity. In the presence of TOPFLASH, cells were co-transfected with pcDNA-CAT (control), indicating the low endogenous b-catenin signaling characteristic of SKBR3 cells; β-catenin, increasing TOPFLASH activity roughly 20-fold; and β-catenin in the presence of cad-11-intact and -variant. The presence of cad-11 reduces β-catenin signaling 5-fold, indicating that cad-11 effectively removes a majority of β-catenin from the signaling pool, likely by stabilizing it at the membrane.

Figure 5 (A) Immunoprecipitation of cadherin-11 was performed from either SK-CAT cells (control) or SK-IV cells (stable SKBR3 expressers of cadherin-11 and variant) with the monoclonal 5B2H5 antibody (2.5μg/ml) in order to verify associational complexes of cadherin-11 with other adherens junction members. Blotting with an anti-p120 antibody (Transduction Labs) reveals that $p120^{ctn}$ is pulled down with cadherin-11. Stripping and reprobing of the original blot proves that wild-type cadherin-11 was in fact precipitated with 5B2H5. In addition, subsequent immunoprecipitations show that both β-catenin and α-catenin can be immunoprecipitated with cadherin-11. Finally, note that both β-catenin and α-catenin appear to have shifted to higher molecular weights in the immune complexes associated with cadherin-11 as compared to the cellular pool seen in the lysate lanes. **L** refers to whole cell lysates, **I** to the immune complex, and **NI** to the non-immune control lane. (B) Immunoprecipitations of either cadherin-11 or E-cadherin in either control cells (MCF7-CAT) or stable expressers of cadherin-11 and variant (MCF7-IV cells). Cells were lysed and cadherin-11 was immunoprecipitated using monoclonal 5B2H5 antibody as described. Blots were probed first for p120 and then stripped and reprobed for β-catenin, α-catenin, and cadherin-11. Cadherin-11 co-precipitates in a complex with all three catenins. E-cadherin (Transduction Labs) was also immunoprecipitated and followed by blotting for the catenins. Note that there is no difference in the amount of any catenin pulled down by E-cadherin in control cells versus expressers, indicating that cadherin-11 does not interfere with E-cadherin association with adherens junction components.

Figure 6 (A) Proliferation rates of SKBR3 cells stably expressing CAT (control), wild-type cadherin-11 (SK-INT) or cadherin-11 and variant (SK-IV) were assessed using the WST-1 assay. Data was plotted relative to mean Day 0 for each cell population to account for differences in plating efficiency. Additionally, both population rates were evaluated as a percentage of their respective CAT controls to

simplify comparison. SKBR3 cells expressing cadherin-11 alone exhibit a modest increase in proliferation rate compared to control cells (approximately 150%), while SKBR3s expressing both forms of the protein exhibit reduced proliferation rates. The difference between cell populations is significant ($p<0.001$), suggesting that the addition of the variant in some way alters or impedes the effects of cadherin-11 on proliferation. (B) Anchorage independent growth was assessed by culturing cells in soft agar as described. SKBR3 populations grow poorly in soft agar, regardless of cadherin-11 expression. (C) The same is true for MCF7 populations, indicating that cadherin-11 expression has little effect on anchorage independent growth in these cells.

Figure 7 Invasive capacity of SKBR3 populations was assessed using a standard Boyden Chamber assay, in which cells invade a matrigel-covered porous membrane to migrate toward chemoattractant-enriched media. Representative membrane fields from each SKBR3 population are depicted on the left, and average number of cells that invaded in a 16-hour period are depicted on the right for both SKBR3 and MCF7 cell populations. Expression of cadherin-11 alone (SK-INT cells) inhibits the invasive activity of SKBR3 cells ($p<0.05$, SK-INT vs. SK-CAT). Cells co-expressing cadherin-11 and variant (SK-IV) invaded twice as much as control cells ($p<0.05$, SK-IV vs. SK-CAT) and ten times faster than cells expressing cadherin-11 alone ($p<0.001$, SK-IV vs. SK-INT). MCF7 cells expressing cadherin-11 and variant (MCF7-IV1) were approximately 5 times more invasive than MCF7 control cells (MCF7-CAT1) ($p<0.05$). Other experiments (data not shown) indicate that all MCF7-IV clones exhibit a similar increase in invasive activity. Error bars represent standard deviation over three wells/experiment.

Figure 8 SKBR3 cells stably expressing cadherin-11 (SK-INT) were transiently transfected with cDNA encoding the variant protein or CAT (control). Twenty-four hours after transfection, cells were trypsinized and assayed for changes in invasive activity using standard Boyden Chambers. (A) SKBR3 cells expressing cadherin-11 and variant (SK-INT + VAR) show a two-fold increase in invasive capacity compared to cells expressing cadherin-11 and CAT control (SK-INT + CAT) when quantified after sixteen hours ($p=0.08$). (B) Above, representative membrane fields from the two populations. Below, after cells were trypsinized, some were plated on coverslips and subsequently fixed and stained for cadherin-11 using the Bussemakers' antibody, which is raised against the extracellular portion of cadherin-11 and should therefore recognize the variant protein (red implies oversaturation of signal). Transfection efficiency only approaches approximately 10%, as discussed in Results.

Figure 9 (A) MCF7 parental cells were transiently transfected with cDNA encoding CAT (control), cadherin-11 or variant. 24 hours later, cells were incubated at 25°C for an additional 24 hours prior to lysis in sample buffer. Whole cell lysates were separated by gel electrophoresis, blotted to nitrocellulose and incubated with ICOS antibody, which is raised against the extracellular portion of cadherin-11 and therefore recognizes both the wild-type and variant forms. Lysate from the variant lane (V) exhibits a strong band at approximately 80 kD, the variant protein (arrow). There is no band at 120kD corresponding to cadherin-11. The cadherin-11 lane (I) does exhibit a strong 120kD band as well as an additional band at 80kD, a presumptive breakdown product. Finally, CAT control cells (C) exhibit neither band. (B) MCF7 cells were transiently transfected with CAT, cadherin-11 or variant cDNA and placed in low serum media to determine whether the variant protein might be secreted. Media was collected 72 hours after transfection and concentrated 40-fold in the presence of protease inhibitors. Western blot analysis of conditioned media failed to detect the variant; IPs were unsuccessful, as well (data not shown). (C) Whole cell lysates of transfected cells verifies the presence of the variant protein. (D) NP-40 insoluble fractions were isolated from cells transiently transfected with the variant cDNA and analyzed by western blot using the ICOS antibody. The variant remains in the insoluble fraction, which contains cytoskeletal elements and associated proteins, as well as extracellular matrix components. (E) SKBR3 cells stably expressing wild-type cadherin-11 were transiently transfected with cDNA encoding the variant or CAT (control). 24 hours after transfection, cells were trypsinized, plated on coverslips, and

subsequently incubated at 25°C for another 24 hours to reduce protein secretion, instead trapping secreted proteins inside the cells. Cells were then fixed, permeabilized, and stained using the Bussemaker antibody, which recognizes the extracellular domain and therefore both proteins. Analysis by confocal microscopy revealed the presence of variant protein in the cytoplasm of SK-INT+VAR but not in SK-INT+ CAT. In addition, this expression was so strong it saturated the confocal image

Figures 1-10

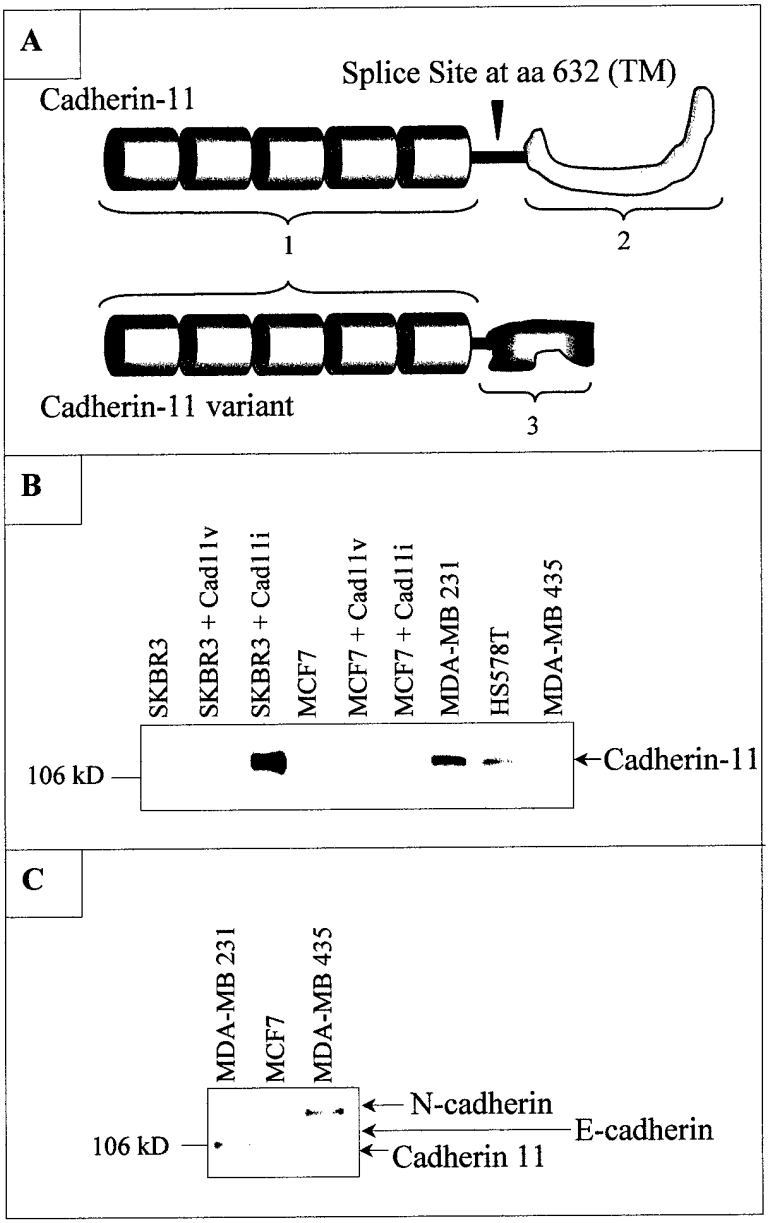

A
Splice Site at aa 632 (TM)
Cadherin-11
1
2
Cadherin-11 variant
3
B
SKBR3
SKBR3 + Cad11v
SKBR3 + Cad11i
MCF7
MCF7 + Cad11v
MCF7 + Cad11i
MDA-MB 231
HS578T
MDA-MB 435
106 kD
Cadherin-11
C
MDA-MB 231
MCF7
MDA-MB 435
N-cadherin
E-cadherin
Cadherin 11
106 kD

Figure 1

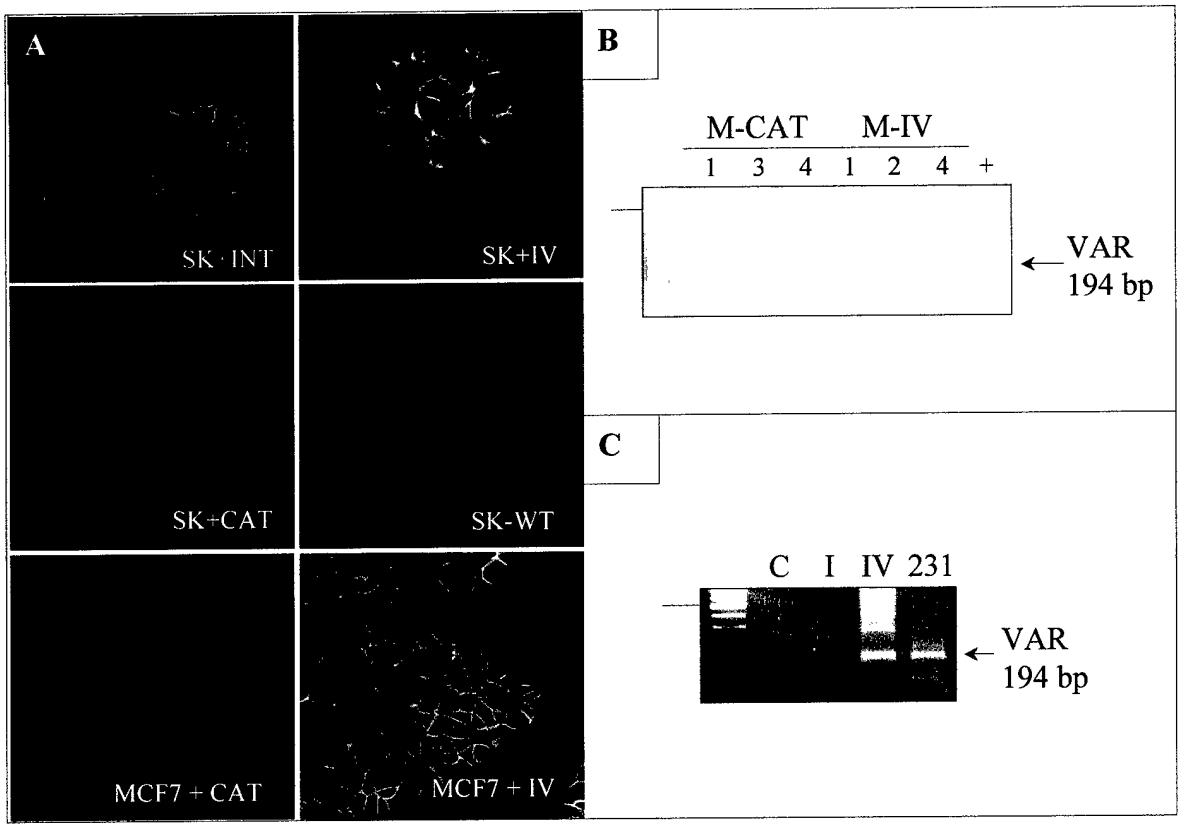

A
SK+INT
SK+IV
SK+CAT
SK-WT
MCF7 + CAT
MCF7 + IV
B
M-CAT
M-IV
1 3 4 1 2 4 +
VAR
194 bp
C
C I IV 231
VAR
194 bp

Figure 2

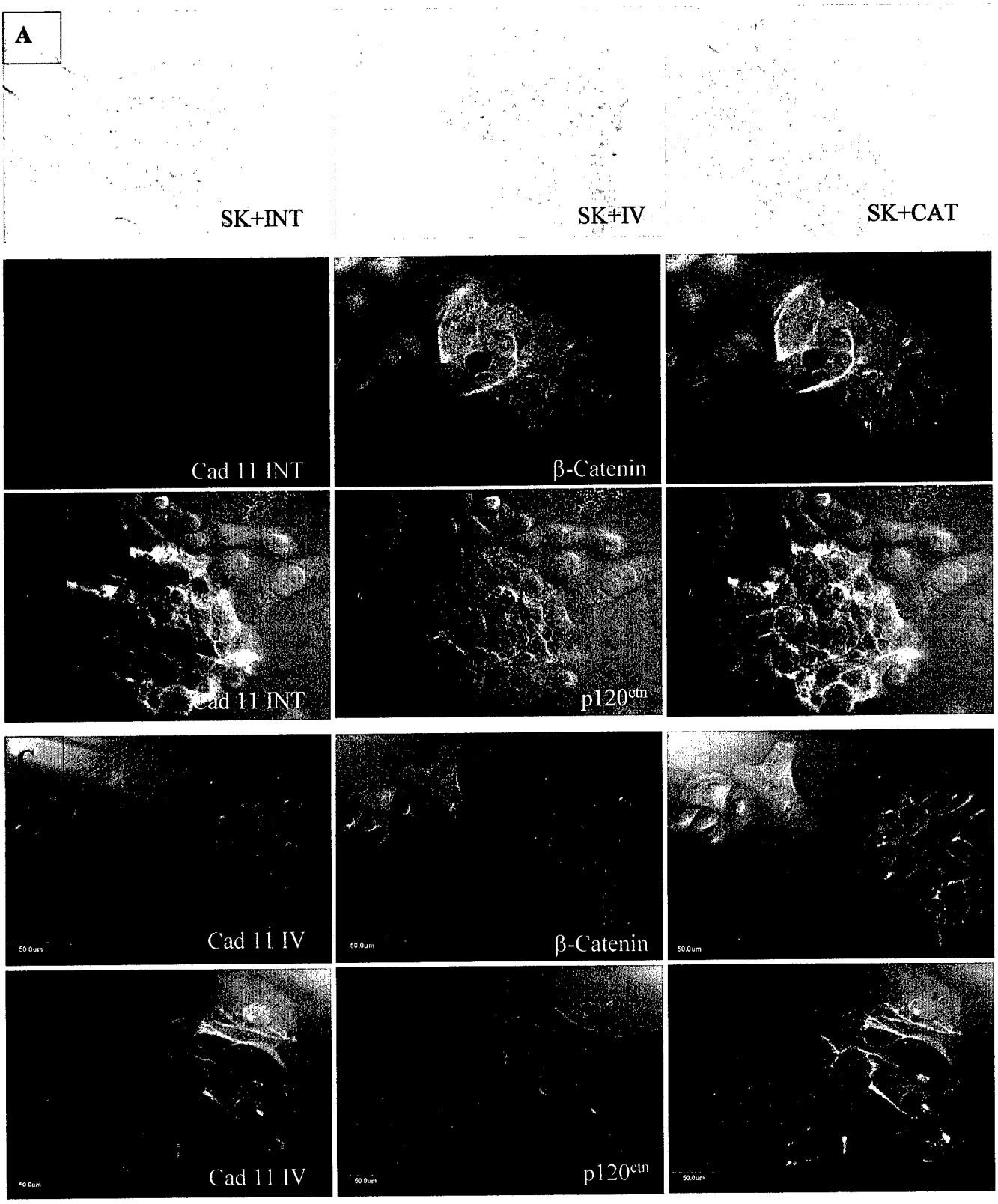

A
SK+INT
SK+IV
SK+CAT
Cad 11 INT
β-Catenin
Cad 11 INT
p120ctn
Cad 11 IV
β-Catenin
Cad 11 IV
p120ctn

Figure 3

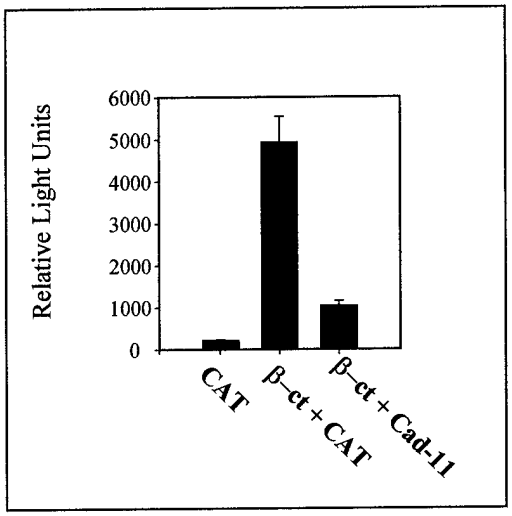

Relative Light Units
6000
5000
4000
3000
2000
1000
0
CAT
β-ct + CAT
β-ct + Cad-11

Figure 4

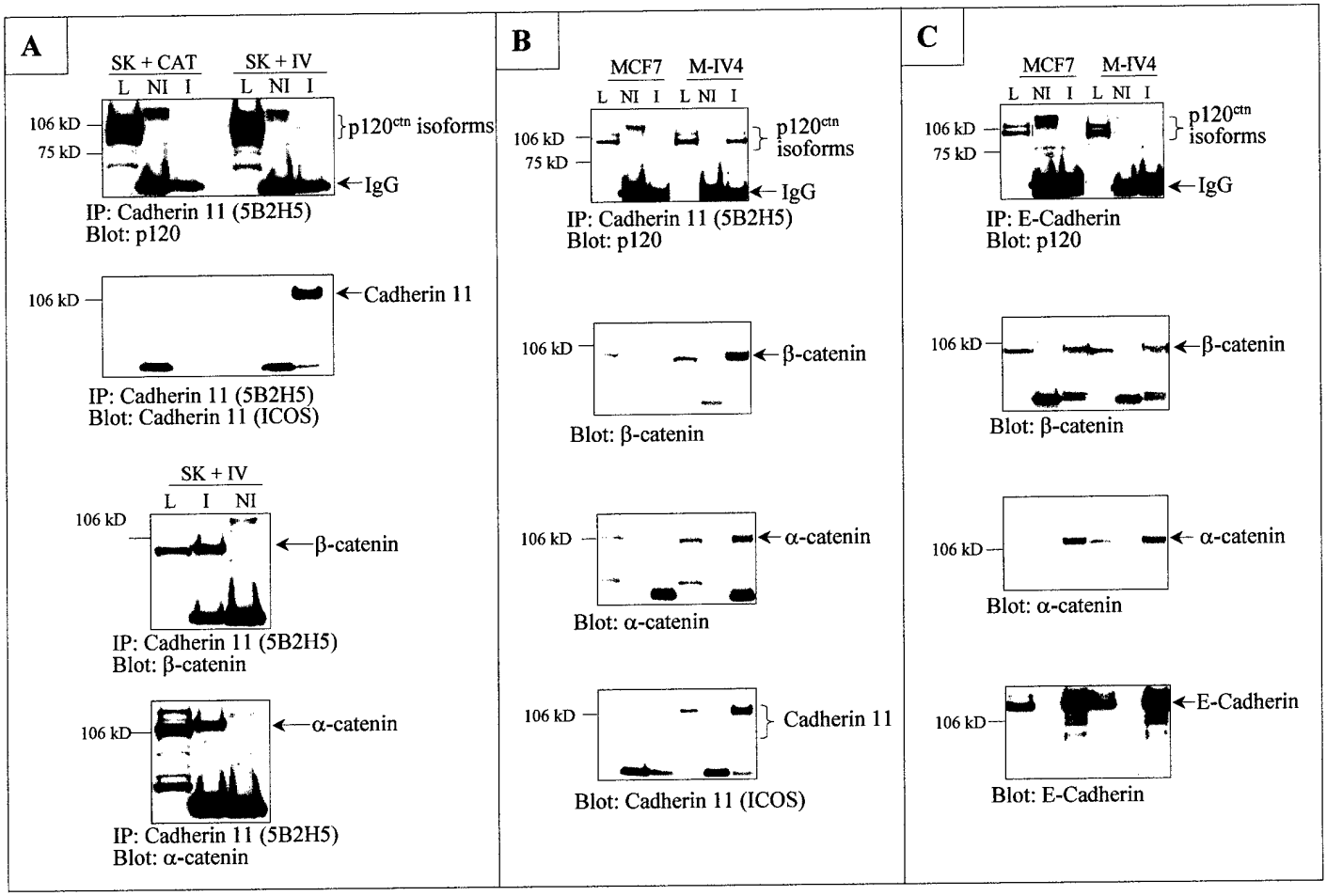

A
SK + CAT
SK + IV
L NI I L NI I
106 kD
75 kD
p120ctn isoforms
IgG
IP: Cadherin 11 (5B2H5)
Blot: p120
106 kD
Cadherin 11
IP: Cadherin 11 (5B2H5)
Blot: Cadherin 11 (ICOS)
SK + IV
L I NI
106 kD
β-catenin
IP: Cadherin 11 (5B2H5)
Blot: β-catenin
106 kD
α-catenin
IP: Cadherin 11 (5B2H5)
Blot: α-catenin
B
MCF7
M-IV4
L NI I L NI I
106 kD
75 kD
p120ctn isoforms
IgG
IP: Cadherin 11 (5B2H5)
Blot: p120
106 kD
β-catenin
Blot: β-catenin
106 kD
α-catenin
Blot: α-catenin
106 kD
Cadherin 11
Blot: Cadherin 11 (ICOS)
C
MCF7
M-IV4
L NI I L NI I
106 kD
75 kD
p120ctn isoforms
IgG
IP: E-Cadherin
Blot: p120
106 kD
β-catenin
Blot: β-catenin
106 kD
α-catenin
Blot: α-catenin
106 kD
E-Cadherin
Blot: E-Cadherin

Figure 5

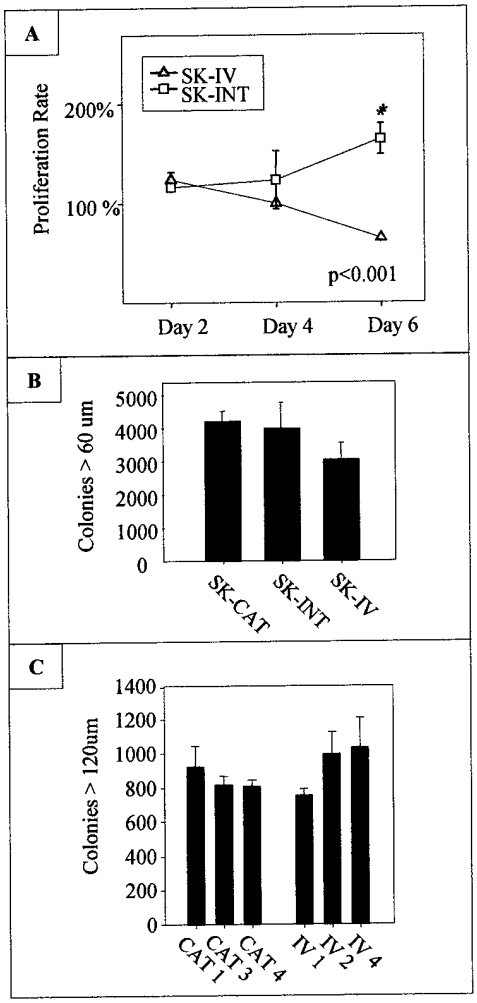

A
SK-IV
SK-INT
Proliferation Rate
200%
100 %
*
p<0.001
Day 2
Day 4
Day 6
B
Colonies > 60 um
5000
4000
3000
2000
1000
0
SK-CAT
SK-INT
SK-IV
C
Colonies > 120um
1400
1200
1000
800
600
400
200
0
CAT 1
CAT 3
CAT 4
IV 1
IV 2
IV 4

Figure 6

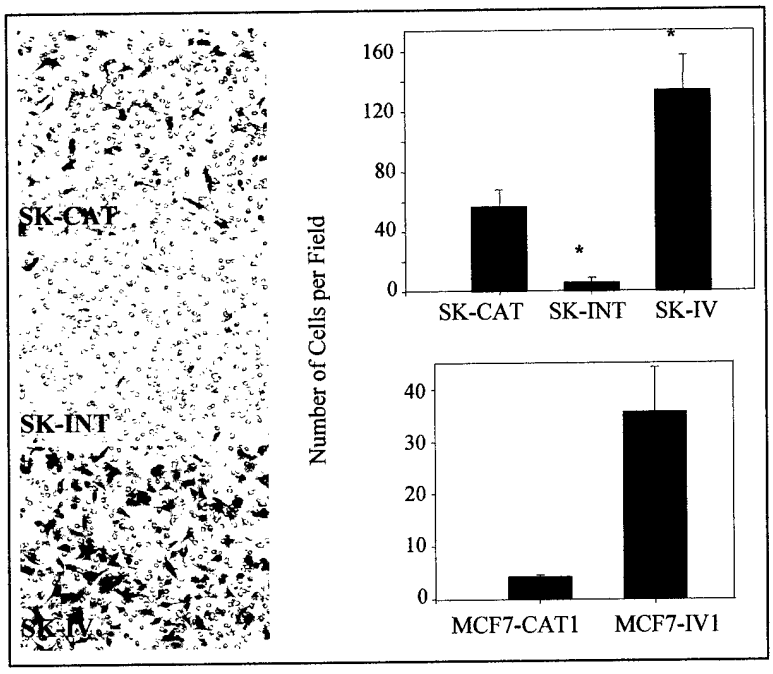

SK-CAT
SK-INT
SK-IV
Number of Cells per Field
160
120
80
40
0
*
*
SK-CAT
SK-INT
SK-IV
40
30
20
10
0
MCF7-CAT1
MCF7-IV1

Figure 7

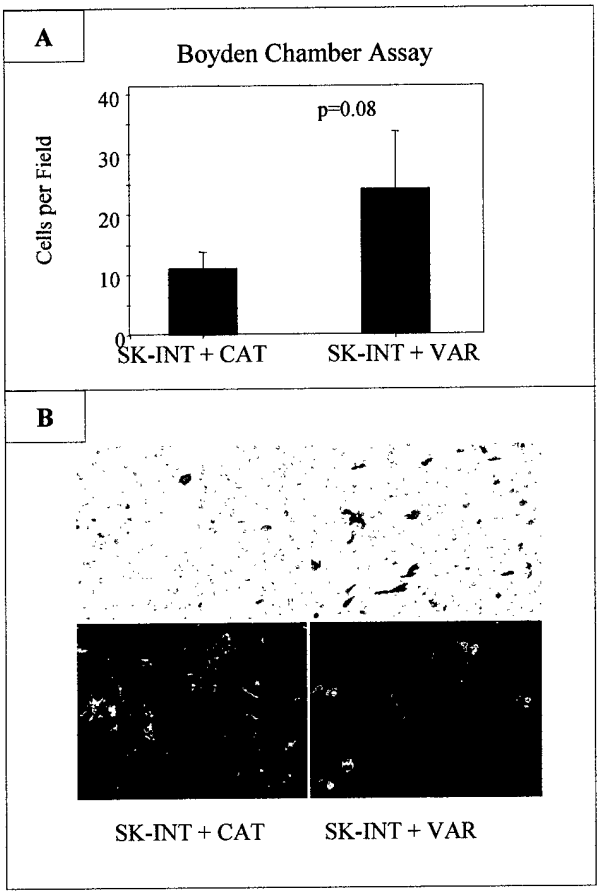

A
Boyden Chamber Assay
Cells per Field
40
30
20
10
0
p=0.08
SK-INT + CAT
SK-INT + VAR
B
SK-INT + CAT
SK-INT + VAR

Figure 8

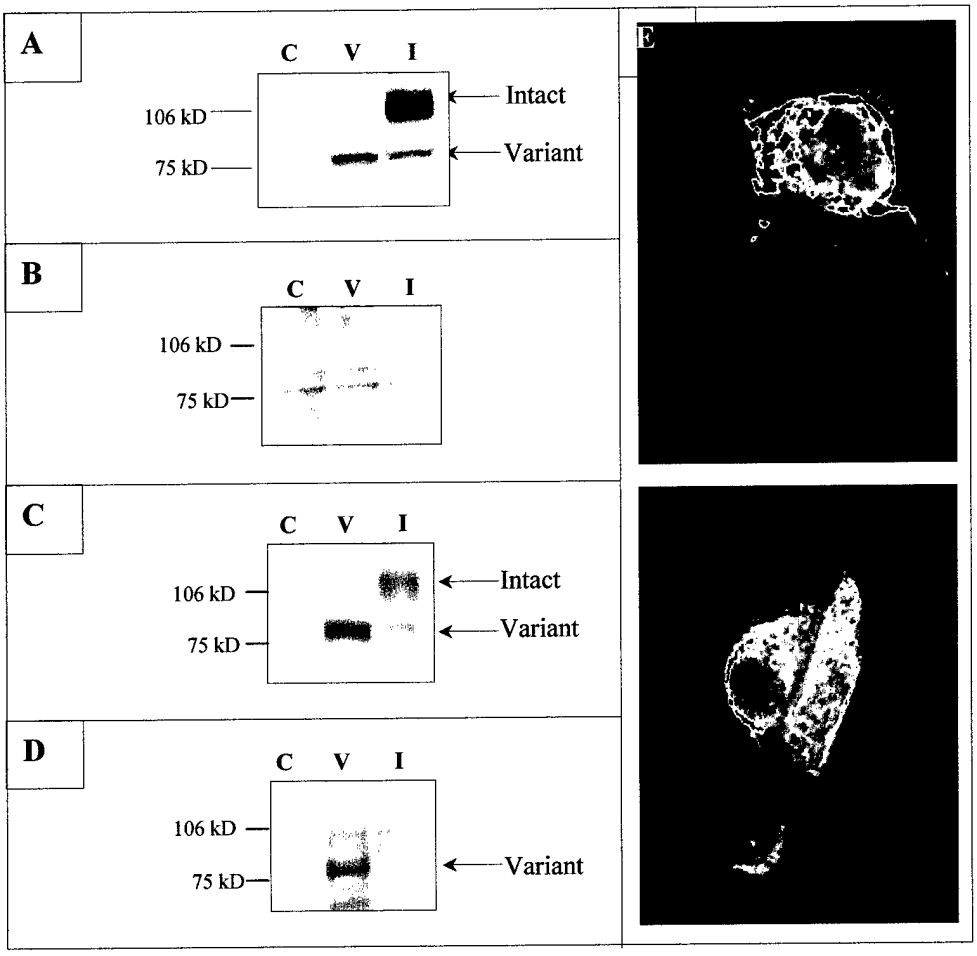

A
C V I
106 kD
75 kD
Intact
Variant
B
C V I
106 kD
75 kD
C
C V I
106 kD
75 kD
Intact
Variant
D
C V I
106 kD
75 kD
Variant
E

Figure 9

Cadherin-11 Ribozyme MDA-231 Clones

5'-
*ATCGTTTGAAA***TTTCGTCCTAACGGACTCATCAG***A*
CAACGGACT-3'
3'-
*TAGCAAACTTT***AAAGCAGGATTGCCTGAGTAGTC***T*
GTTGCCTGA-5'

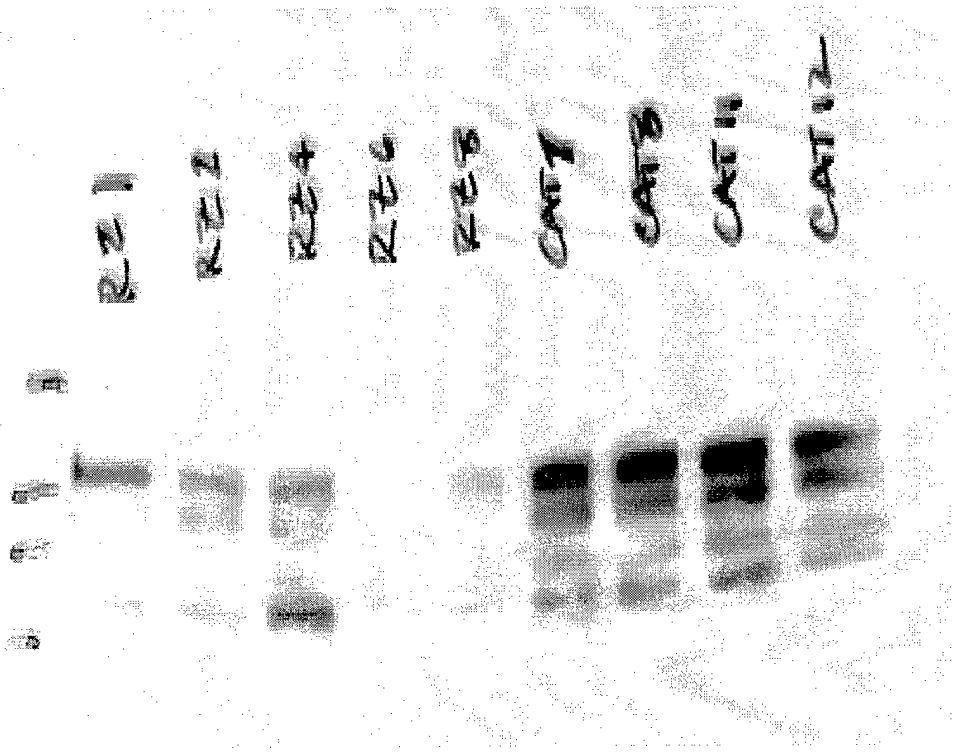
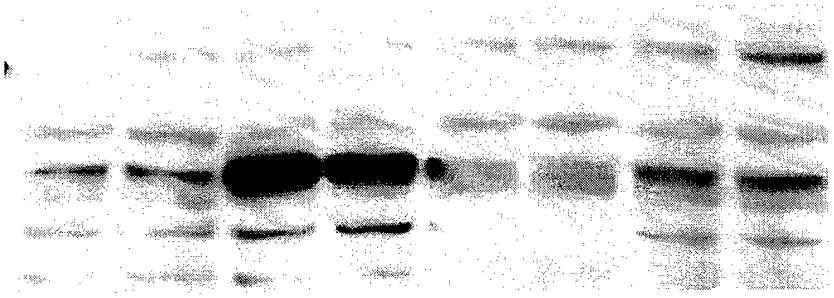


Figure 10

Rb2



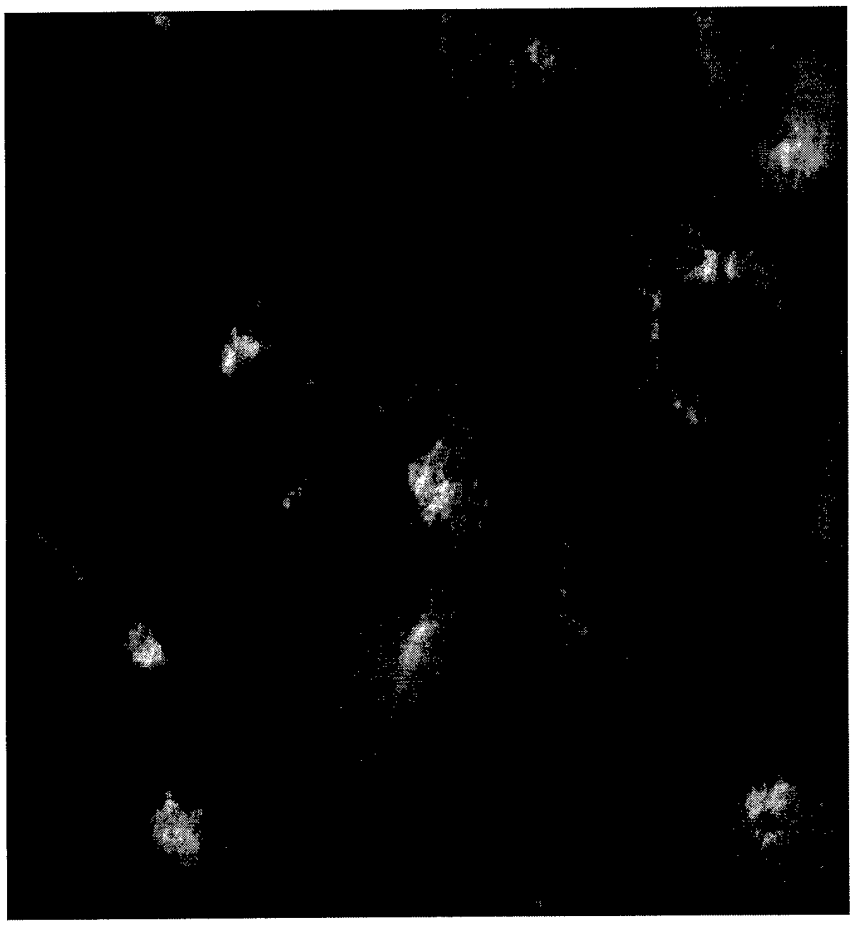


Figure 9